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Original scientific paper

Synthesis and high *in vitro* cytotoxicity of some (*S*,*S*)-ethylenediamine-*N*,*N*'-di-2-propanoate dihydrochloride esters

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Abstract. A novel (*S*,*S*)-R_2eddip ester, *O*,*O*′-diisopentyl-(*S*,*S*)-ethylenediamine--*N*,*N*′-di-2-propanoate dihydrochloride (**1**) was synthesized and characterized by IR, ^{1}H- and ^{13}C-NMR spectroscopy, mass spectroscopy and elemental analysis. *In vitro* antitumor action of **1**, and two more R_2eddip esters, dialkyl (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate dihydrochlorides, obtained before (alkyl = *n*-Bu or *n*-Pe, **2** and **3**, respectively), was determined against cervix adenocarcinoma (HeLa), human melanoma (Fem-x), human chronic myelogenous leukemia (K562) cells, and a non-cancerous cell line human embryonic lung fibroblast (MRC-5), using the microculture tetrazolium test MTT assay. Esters **1**–**3** showed higher cytotoxicity and better selectivity in comparison to cisplatin, used as reference compound. The highest activity was expressed by **1**, with IC_{50}(Fem-x) value of 1.51±0.09 μM.

Keywords: R_2edda-type esters; characterization; cytotoxicity; selectivity.

INTRODUCTION

Cancer is one of the most widespread and feared diseases in the world because it is very difficult to cure as cancer cells are not foreign to the body but are simply subtly mutated forms of normal human cells that multiply without control.[1–3]

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The majority of drugs used for the treatment of cancer today are cytotoxic (cell-killing) drugs that work by interfering in some way with the operation of the cell's DNA.

Over the last fifty years, about 500,000 natural and synthetic chemical compounds have been tested for their anticancer activity, but only about 25 of these are in wide use today.[4] A major challenge is to design new drugs that will be more selective for cancer cells, and thus have fewer side effects.

Cisplatin showed potent antitumor activity in the 70's and nowadays it is routinely used in the treatment of many types of cancers.[5–9] Unfortunately, it also causes severe side-effects, such as nephro-, oto- and neuro-toxicity.[10,11] Many platinum complexes were synthesized with hope for better pharmacological properties.[12–14] Besides cisplatin, only carboplatin and oxaliplatin are in worldwide clinical use.[15]

During the last decade, our research group has been engaged in the synthesis, characterization and investigation of the antitumor activity of platinum(II) and platinum(IV) complexes with *N*,*N*′ bidentate ligands, R_2edda-type esters.[16–26] These ligands, obtained by structural variations of the aminocarboxylato arms and alkyl groups of the ester moiety (normal, branched chains, rings), yielded a large library of such molecules (Fig. 1). In most cases, high cytotoxic action was accomplished when coordinating to platinum ions, but some of the esters, in fact, showed serious activity themselves.[27,28]

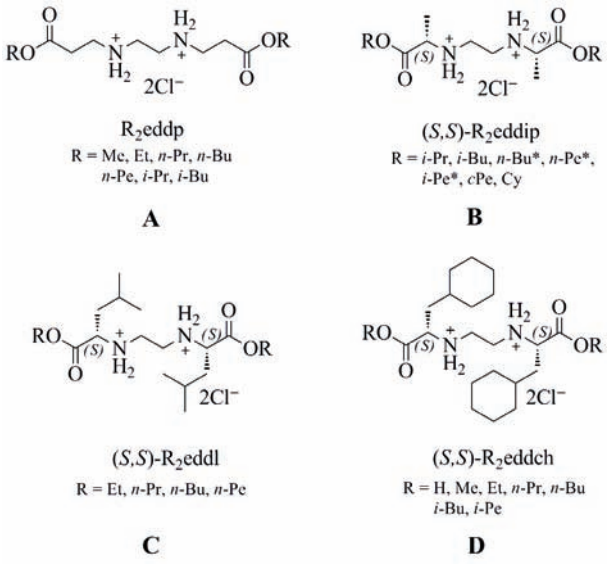


Fig. 1. R_2edda-type esters (* – esters investigated in this work).

These compounds (R_2edda-type esters, R = alkyl; eddp = ethylenediamne-*N*,*N*′-di-3-propanoate, Fig. 1, A; (*S*,*S*)-eddip = (*S*,*S*)-ethylenediamne-*N*,*N*′-di-2--propanoate, Fig. 1, B; (*S*,*S*)-eddl = (*S*,*S*)-*N*,*N*′-1,2-ethanediylbisleucinate, Fig. 1, C; (*S*,*S*)-eddch = (*S*,*S*)-α,α'-(1,2-ethanediyldiimino)biscyclohexanepropanoate, Fig. 1, D) were tested against various cancer lines and normal cells and moderate or low activity was found,[20,21,23,24,29,30] except for (*S*,*S*)-Et_2eddch that had the lowest IC_{50} values *ca*. 11 μM against HL-60, U251, C6, L929 and B16 cell lines.[27,28]

Herein, the synthesis, characterization and antiproliferative activity of one novel R_2edda-type ester: diisopentyl (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate dihydrochloride (**1**) is reported. This newly synthesized compound, along with already reported[22] di(*n*-butyl) (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate dihydrochloride (**2**) and di(*n*-pentyl) (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate dihydrochloride (**3**), were tested against cervix adenocarcinoma cell line (HeLa), human melanoma (Fem-x), human chronic myelogenous leukemia (K562) cells, and a non-cancerous cells MRC-5 (human embryonic lung fibroblast), with the aim of assessing their cytotoxic actions.

EXPERIMENTAL

Material and methods

All reagents were of analytical grade. (*S*,*S*)-Ethylenediamine-*N*,*N*′-di-2-propanoic acid hydrochloride, (*S*,*S*)-H_2eddip·HCl, was prepared by a standard procedure.[31]

Elemental analyses were realized on an Elemental Vario EL III microanalyzer. The infrared spectra were recorded using a Nicolet 6700 FT-IR spectrophotometer (Thermo Scientific), ATR technique (smart accessory orbit with diamond crystal) in the range of 4000–400 cm^{-1}. The NMR spectra were recorded on Varian Gemini 200 and Bruker Avance III 500 spectrometers. The chemical shifts for the ^{1}H- and ^{13}C-NMR spectra were referenced to residual ^{1}H and ^{13}C present in DMSO-d_6. The mass spectra were recorded with a 6210 Time-of-Flight LC–MS instrument (G1969A, Agilent Technologies). An Agilent Technologies 1200 series HPLC instrument (Agilent Technologies, Waldbronn, Germany) equipped with auto sampler, binary pump, DAD detector and ZDV cell, was used for the introduction of the sample dissolved in methanol into the mass spectrometer. As the mobile phase 0.2 % formic acid in water/acetonitrile (*V*/*V* = 50/50) at a flow of 0.15 mL min^{-1} was used. The mass spectra were recorded in the positive mode under the following conditions: capillary voltage 2500 V, gas temperature 250 °C, drying gas flow 7 L min^{-1}, nebulizer pressure 30 psig, fragmentor voltage 50 V, mass range 100–1500 *m*/*z*. For data collection and interpretation, MassHunter Workstation software was used.

An automatic polarimeter AUTOPOL® IV, Rudolph Research Analytical, a sodium lamp (589 nm) with a 1-dm cell was used to determine the specific rotation.

Synthesis of (S,S)-(i-*Pe*)$_2$*eddip 2HCl* (***1***)

The R_2edda-type ester, **1**, was prepared using the esterification reaction previously described for similar compounds.[18,32] Thionyl chloride (4 ml, 55 mmol) was introduced into a flask containing 40 ml of ice-cooled isopentyl alcohol (3-methyl-1-butanol), (*t* = 0 °C, anhydrous conditions) during 1 h through a dropping funnel. Subsequently, 1.50 g (5.41 mmol) of

(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoic acid hydrochloride, (*S*,*S*)-H_2eddp·HCl, was added to the flask and the suspension was refluxed for 16 h at ≈130 °C. The mixture was filtered hot, immediately after reflux and the filtrate was left for a few days at 4 °C yielding the white product.

Yield: 1.22 g, 54 %. Anal. Calcd. for $C_{18}H_{38}N_2O_4Cl_2 \cdot 0.75H_2O$: C, 50.17; H, 9.23; N, 6.50 %. Found: C, 50.07; H, 8.77; N, 6.44 %; IR (cm^{-1}): 2961, 2862, 2636, 2598, 2416, 1733, 1236, 1160, 803; ^{1}H-NMR (500 MHz, DMSO-d_6, δ / ppm): 0.90 (12H, *d*, $^3J_{H,H}$ = 6.5 Hz, CH_3−*i*-Pe), 1.48–1.55 (10H, *m*, CH_2−*i*-Pe, CH_3), 1.68 (2H, *sep*, $^3J_{H,H}$ = 6.5 Hz, CH−*i*-Pe), 3.31–3.47 (4H, *m*, CH_2-(en)), 4.03 (2H, *q*, $^3J_{H,H}$ = 4.0 Hz, CH), 4.16–4.28 (4H, *m*, CH_2O−*i*-Pe), 10.09 (4H, *brs*, NH_2^+); ^{13}C-NMR (50 MHz, DMSO-d_6, δ / pm): 14.4 (CH_3), 22.4 (CH_3−*i*-Pe), 24.5 (CH−*i*-Pe), 36.7 (CH_2−*i*-Pe), 54.5 (CH_2-(en)) 64.6 (CH), 70.2 (CH_2O−*i*-Pe), 169.4 (COO−*i*-Pe); ESI−MS (CH_3OH), positive mode: Calcd. 345.27478. Found *m*/*z*: 345.27449 $[M–2Cl–H]^+$; $[\alpha]_D^{20}$ = –15° (CH_3OH, 1.1 mg/mL).

Biological experiments

Cell lines. Cervix adenocarcinoma cell line (HeLa), human melanoma (Fem-x), human chronic myelogenous leukemia (K562) cells, and a non-cancerous cell line, MRC-5 (human embryonic lung fibroblast) were grown in RPMI-1640 medium (Sigma Aldrich, St. Louis, MO, USA). The media were supplemented with 10 % fetal bovine serum, L-glutamine, and penicillin–streptomycin (Sigma Aldrich, St. Louis, MO, USA).

Treatment of cell lines. The target cells HeLa (2000 cells per well), Fem-x (5000 cells per well), K562 (5000 cells per well), and non-cancerous MRC-5 (5000 cells per well) were seeded into wells of a 96-well flat-bottomed microtitre plate. Twenty-four hours later, after cell adherence, different concentrations of the investigated compounds were added to the wells, except for the control cells to which the nutrient medium only was added. The final chosen concentrations range was 1–100 μM (1.0, 8.25, 16.5, 33.0 and 100.0 μM). The final concentration of DMSO solvent never exceeded 0.5 %, which was non-toxic to the cells. Especially, compounds were applied to the suspension of K562 cells 2 h after cell seeding. All concentrations were set up in triplicate. Nutrient medium with corresponding concentrations of investigated compounds, but without cells, was used as a blank, also in triplicate. The cultures were incubated for 72 h.

Determination of cell survival. The effect of the prepared compounds on cancer cell survival was determined by the microculture tetrazolium test (MTT) according to Mosmann[33] with modification by Ohno and Abe[34] 72 h after addition of the compounds, as described earlier. Briefly, 20 μl of methylthiazoletetrazolium bromide, 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution (5 mg ml^{-1} phosphate-buffered saline) was added to each well. Samples were incubated for a further 4 h at 37 °C in a humidified atmosphere of 95 vol. % air/5 vol. % CO_2. Then 100 μL of 100 g L^{-1} sodium dodecyl sulfate solution was added to extract the insoluble product formazan resulting from the conversion of the MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of the absorbance of light, which was read in an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm. The absorbance (*A*) at 570 nm was measured 24 h later. To determine cell survival (%), the *A* of a sample with cells grown in the presence of various concentrations of the investigated compounds was divided by the control optical density (the *A* of control cells grown only in nutrient medium) and multiplied by 100. Absorbance of the blank was always subtracted from the A of the corresponding sample with target cells. The IC_{50} is defined as the concentration of an agent inhibiting cell survival by 50 % compared

with an untreated control. Cisplatin was used as the positive control. All experiments were performed in triplicate.

The selectivity index. The selectivity index (*SI*) is defined as the ratio of the IC_{50} obtained from the experiments on normal cells to that obtained on cancer cells. As the selectivity index (*SI*) demonstrates the differential activity of a pure compound, the greater the SI value is, the more selective is the compound.[35,36]

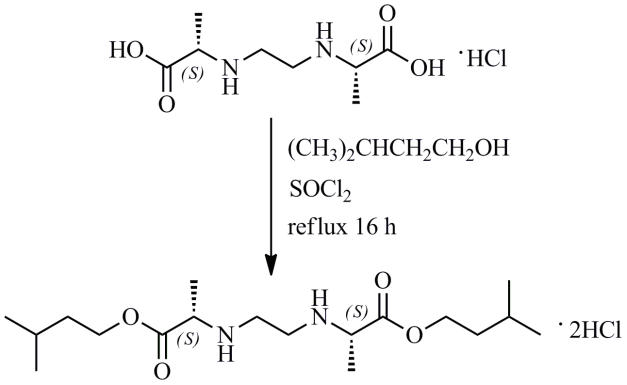
Statistical analysis

Results are presented as the mean ± standard deviation (*SD*) of triplicate observations from the representative of three experiments. The significance of the difference between treatments and control was analyzed by ANOVA followed by the Student-Newman-Keuls test $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Spectroscopic studies

In the reaction of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoic acid hydrochloride, (*S*,*S*)-H$_2$eddip·HCl (crystal structure was described recently),[37] with absolute isopentyl alcohol (3-methyl-1-butanol) in the presence of thionyl chloride, diisopentyl (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate dihydrochloride (**1**) was obtained (Scheme 1).



Scheme 1. Synthesis of (*S*,*S*)-(*i*-Pe)$_2$eddip·2HCl (**1**).

The IR spectrum of **1** showed characteristic absorption bands for this class of compounds.[22,23,28] A strong absorption stretching band ν(C=O) was found at 1733 cm^{-1}, while a band arising from ν(C−O) appeared at 1236 cm^{-1}. Asymmetric CH_3 stretching vibrations of medium intensity were found at ν(CH_3) 2961 cm^{-1} and 2862 cm^{-1}. Furthermore, an asymmetric ν(C−N) stretching vibration was found at 803 cm^{-1}.

In the ^{1}H-NMR spectrum resonances of methyl hydrogen atoms from the isopentyl moieties were found at 0.90 ppm as a doublet. Chemical shifts of the hydrogen atoms from isopentyl-CH_2 groups, overlapped with CH_3 protons

(L-alanine moiety), were found as a multiplet at 1.48–1.55 ppm, while CH_2 protons belonging to carbon atoms attached to ester oxygen could be seen at 4.21 ppm. Hydrogen atoms belonging to tertiary carbon atoms (isopentyl groups) were found as a septet at 1.69 ppm. The CH protons from the L-alanine moiety were detected at 4.03 ppm. The chemical shifts assignable to −N−C**H**$_2$−C**H**$_2$−N− protons were found at 3.31–3.47 ppm. Resonances of hydrogen atoms belonging to secondary ammonium groups were found at 10.09 ppm as broad singlets.

In the ^{13}C-NMR spectrum, the carbonyl carbon atoms peak was found at 169.4 ppm as expected for this class of compounds.[23,29] The ethylenediamine carbon atoms resonated at 54.5 ppm. The carbon atoms bonded to the ester oxygens were detected at 70.2, but resonances of all other carbon atoms (from isopentyl and L-alanine moieties) were found below 40 ppm, as expected.[22] The high resolution mass spectrum (positive mode) of **1** showed the presence of $[M–2Cl–H]^+$.

In vitro *studies*

Cytotoxicity. The *in vitro* cytotoxicity of esters **1–3** toward HeLa cervix adenocarcinoma, Fem-x human melanoma, K562 human chronic myelogenous leukemia cell lines and non-cancerous MRC-5 human embryonic lung fibroblast cells were determined by MTT assay. Cisplatin was used as a reference. The results are summarized in Table I, while Fig. 2 depicts the cytotoxic curves from the MTT assay showing the survival of target cells grown for 72 h in the presence of increasing concentrations of **1–3**.

TABLE I. IC_{50} values (μM) for **1**–**3** and cisplatin on the malignant HeLa, Fem-x and K562 cell lines and non-cancerous MRC-5 normal cells; the IC_{50} values are expressed as the mean±*SD* determined from the results of the MTT assay in three independent experiments

Compound	Cell line			
	HeLa	Fem-x	K562	MRC-5
1	2.01±0.19	1.51±0.09	5.22±0.55	51.09±1.06
2	2.22±0.81	2.25±0.91	3.27±0.58	>100
3	1.75±0.44	2.31±0.79	2.13±1.45	53.79±0.84
Cisplatin	2.10±0.20	5.51±0.31	5.54±1.03	14.21±1.54

The investigated compounds demonstrated a remarkable cytotoxic activity, as the IC_{50} values are in range from 1.51 to 5.22 μM against all the tested malignant cell lines. The IC_{50} values of these compounds against all cancer cell lines were in the micromolar range, similar to or better than those of the antitumor drug cisplatin. Namely, **1**–**3** and cisplatin showed no significant difference in *in vitro* activity against HeLa cells. Furthermore, all compounds exhibited significantly higher activity than cisplatin against K562 and Fem-x cell lines, except complex **1** against K562 cells.

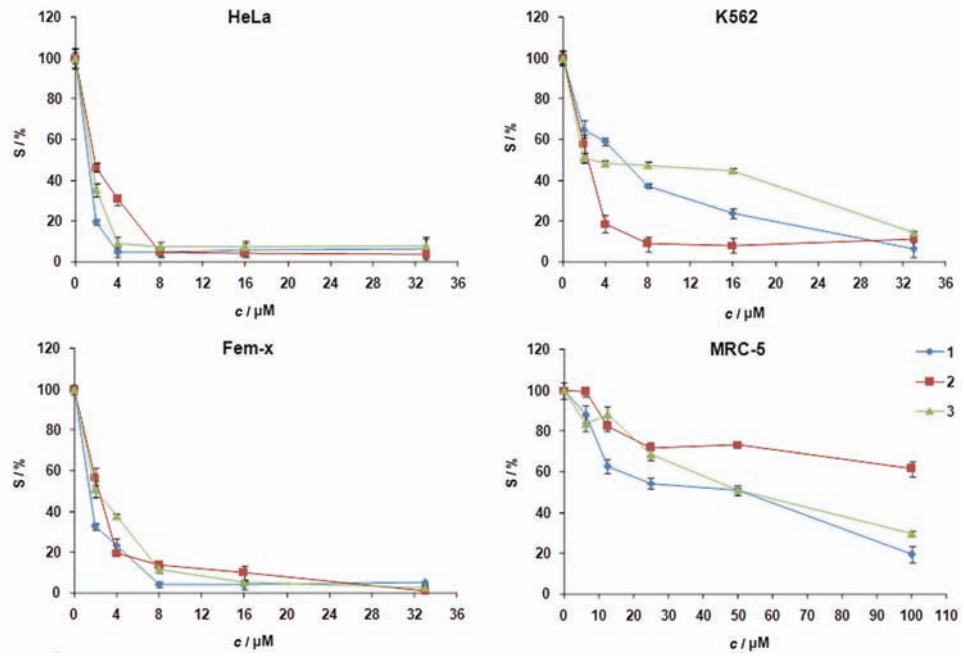


Fig. 2. Representative graphs show the survival of HeLa, K562, Fem-x, and MRC-5 cells grown for 72 h in the presence of increasing concentrations of **1**–**3**.

These findings indicate that the described compounds are extremely important for the future screening of their biological activity. In addition, from the obtained results, it could be concluded that compounds **1–3** show very slight differences in their cytotoxic effects against the tested cancer cells. Obviously, the presence of the isopentyl, *n*-butyl or *n*-pentyl groups in the structure of these compounds does not lead to significant differences in their activities.

Selectivity. Against the non-cancerous lung fibroblasts (MRC-5), all of the compounds exhibited a significantly weaker activity compared to cisplatin. The toxicity of compounds **1** and **3** in the lung fibroblasts was almost four times lower than that of cisplatin (Table I), and ten to fifty times weaker in the healthy cells compared to all the tested cancerous cells (Table II). Remarkably, compound **2** showed no cytotoxicity to normal non-cancerogenic MRC-5 cells (IC_{50} > 100 μM). The present *in vitro* experiments showed that compounds **1**–**3** express very high cytotoxic activity to cancerous cells with great selectivity (Table II), whereby compound **2** had no cytotoxic activity against mammalian normal cells (in the investigated concentration range). The effects of these compounds towards cancer and normal cells indicate to the necessity for further studies with *in vitro* and/or *in vivo* tests.

TABLE II. Selectivity index ($SI = IC_{50}$(MRC-5)/IC_{50}(cell line))

Compound	Cell		
	HeLa	Fem-x	K562
1	25.42±0.19	33.83±2.14	9.79±1.05
2	> 45.05	> 44.44	> 30.58
3	30.74±7.74	23.29±7.97	25.25±17.20
Cisplatin	6.77±0.98	2.58±0.31	2.56±0.55

CONCLUSIONS

The R_2eddip ester, diisopentyl (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate dihydrochloride (**1**) was synthesized. The compound was characterized by IR, ^{1}H- and ^{13}C-NMR spectroscopy, mass spectrometry and by elemental analysis. This novel R_2eddip·2HCl, **1**, ester along with earlier reported di(*n*-butyl) (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate dihydrochloride (**2**) and di(*n*-pentyl) (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate dihydrochloride (**3**) were tested against cervix adenocarcinoma cell line (HeLa), human melanoma (Fem-x), human chronic myelogenous leukemia (K562) cells and the non-cancerous cell line human embryonic lung fibroblast (MRC-5), using the MTT assay. Esters **1**–**3** showed similar or higher cytotoxicity, but much better selectivity, in comparison to cisplatin. (*S*,*S*)-(*i*-Pe)$_2$eddip (**1**) expressed the highest activity against Fem-x cells (IC_{50} = 1.51±0.09 μM).

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И З В О Д

СИНТЕЗА И ВИСОКА *IN VITRO* ЦИТОТОКСИЧНОСТ НЕКИХ (*S*,*S*)-ЕТИЛЕНДИАМИН-*N*,*N*′-ДИ-2-ПРОПАНОАТ-ДИХИДРОХЛОРИД ЕСТАРА

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Нов (*S*,*S*)-R_2eddip естар, *O*,*O*′-диизопентил-(*S*,*S*)-етилендиамин-*N*,*N*′-ди-2-пропаноат-дихидрохлорид (**1**), синтетисан је и окарактерисан уз помоћ IR, NMR и масене спектрометрије и елементалне анализе. *In vitro* антитуморска активност једињења **1**, и још два R_2eddip естра, *O*,*O*′-диалкил-(*S*,*S*)-етилендиамин-*N*,*N*′-ди-2-пропаноат-дихидрохлорида, који су раније објављени (алкил = *n*-Bu или *n*-Pe, **2** и **3**, редом) испитивани су на хуманим ћелијским линијама аденокарцинома материце (HeLa), малигног меланома (Fem-x), и мијелоидне леукемије (K562), као и на нормалној ћелијској линији MRC-5 (фетални плућни фибробласти), уз помоћ MTT теста. Естри **1**–**3** су показали високу цитотоксичност и бољу селективност у поређењу са цисплатином која је коришћена као

референтна супстанца. Највећу активност је показао естар **1** са IC_{50}(Fem-x) вредношћу 1,51±0,09 μM.

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